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This is the author's manuscript

Original Citation:

Availability:

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This is the author's final version of the contribution published as:

M. Gelardi; A. Vizzini; E. Ercole; S. Voyron; J.Z. Sun; X.Z. Liu. *Boletus sinopulverulentus*, a new species from Shaanxi Province (central China), and notes on *Boletus* and *Xerocomus*. SYDOWIA. 65 (1) pp: 45-57.

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Boletus sinopulverulentus*, a new species from Shaanxi Province (central China) and notes on *Boletus* and *Xerocomus

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Gelardi M., Vizzini A., Ercole E., Voyron S., Sun J.-Z., Liu X.-Z. (2013) *Boletus sinopulverulentus*, a new species from Shaanxi Province (central China) and notes on *Boletus* and *Xerocomus*. – *Sydowia* 65 (1): 45–57.

Boletus sinopulverulentus, a new species discovered in Shaanxi Province (central China), is formally described and illustrated. A detailed morphological description is provided and accompanied by line-drawings of the main anatomical features, a colour illustration of fresh material in habitat and the pertinent supporting molecular data (ITS sequences analysis). *Boletus sinopulverulentus* belongs to the *B. pulverulentus* species complex. The diagnostic traits of the species allied to the new taxon are also discussed for comparison. Finally, based on molecular analysis the taxonomy of *Boletus* and *Xerocomus* is briefly discussed.

Keywords: *Boletaceae*, *Boletus pulverulentus*, Chinese ectomycorrhizal fungi, molecular phylogeny, taxonomy.

The fungal biodiversity of Qinling Mountains in Shaanxi Province (China) appears to be largely understudied and only few boletes have so far been reported from that area (Tian *et al.* 1995, Mao *et al.* 1997, Tian *et al.* 2000, Shen *et al.* 2008). Recently, during extensive fieldwork in the Qinling range focusing on recording boletoid taxa, the first author came across a noteworthy collection belonging to sect. *Subpruinosi* Fr. in the genus *Boletus* L. s.l. Careful examination of macro- and microscopical characters supported by molecular analysis confirmed that this collection represents a new species, described here as *Boletus sinopulverulentus*, which is phylogenetically closely related to *B. pulverulentus* Opat. In addition, the morphologically based taxonomy of *Boletus* and *Xerocomus* Quél. by Muñoz (2005) and Šutara (1991, 2005, 2008) was critically tested using molecular data.

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Materials and methods

Morphology

Macroscopic description, habitat notations and associated plant communities were based upon detailed field notes of fresh basidiomata. Colour terms in capital letters (e.g. Auburn, Plate II) are those of Ridgway (1912). Micromorphological features were observed on dried material; sections were soaked in water, 5 % potassium hydroxide (KOH) or in ammoniacal Congo Red. The observation of the structures and measurement of the anatomical features was performed mounting the preparation in ammoniacal Congo Red, the colour and the amount of pigmentation was described after examination in water and 5 % KOH. Measurements were made at 1000x magnification with a calibrated ocular micrometer. Spore dimensions are taken from the hymenophore and given as (minimum) average \pm standard deviation (maximum), Q= average quotient (length/width ratio) \pm standard deviation, while average spore volume was approximately estimated as a rotation ellipsoid ($V = 4/3 * (\text{length}/2) * ((\text{width}/2) * \text{width}) * \pi/2 \pm \text{standard deviation}$). The notation [50,1,1] indicates that measurements were made on 50 spores from one sample of one collection. The width of each basidium was measured at the widest part, and the length was measured from the apex (sterigmata excluded) to the basal septum. Metachromatic, cyanophilic and iodine reactions were also tested staining the spores in Brilliant Cresyl blue, Cotton blue and Melzer's reagent, respectively. Anatomical features were observed with a Nikon Eclipse E200 optical light microscope and all line-drawings of microstructures were made from rehydrated material. The type collection examined in this study was deposited in HMAS. Herbarium acronyms follow Thiers (2012) except "MG" and "TL" that refer to the personal herbaria of Matteo Gelardi and Tomaso Lezzi. Author citations follow the Index Fungorum-Authors of Fungal Names (<http://www.indexfungorum.org/authors/fungalnames.htm>).

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was isolated from 1 mg of one herbarium specimen (HMAS 266894) using the DNeasy Plant Mini Kit (Qiagen, Milan Italy). Universal primers ITS1f/ITS4 were used for the ITS region amplification (White *et al.* 1990, Gardes & Bruns 1993). Amplification reactions were performed in PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 25 μ l reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1xPCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 μ M of each primer, 2.5 mM MgCl₂, 0.25 mM of each dNTP, 0.5 unit of Taq polymerase (Promega). The PCR program was as follows: 3 min at 95 °C for 1 cycle; 30 s at 94 °C, 45 s at 50 °C, 2 min at 72 °C for 35 cycles, 10 min at 72 °C for 1 cycle. PCR products were resolved on a 1.0 % agarose gel and visualized by staining with ethidium bromide. PCR products were purified and sequenced by

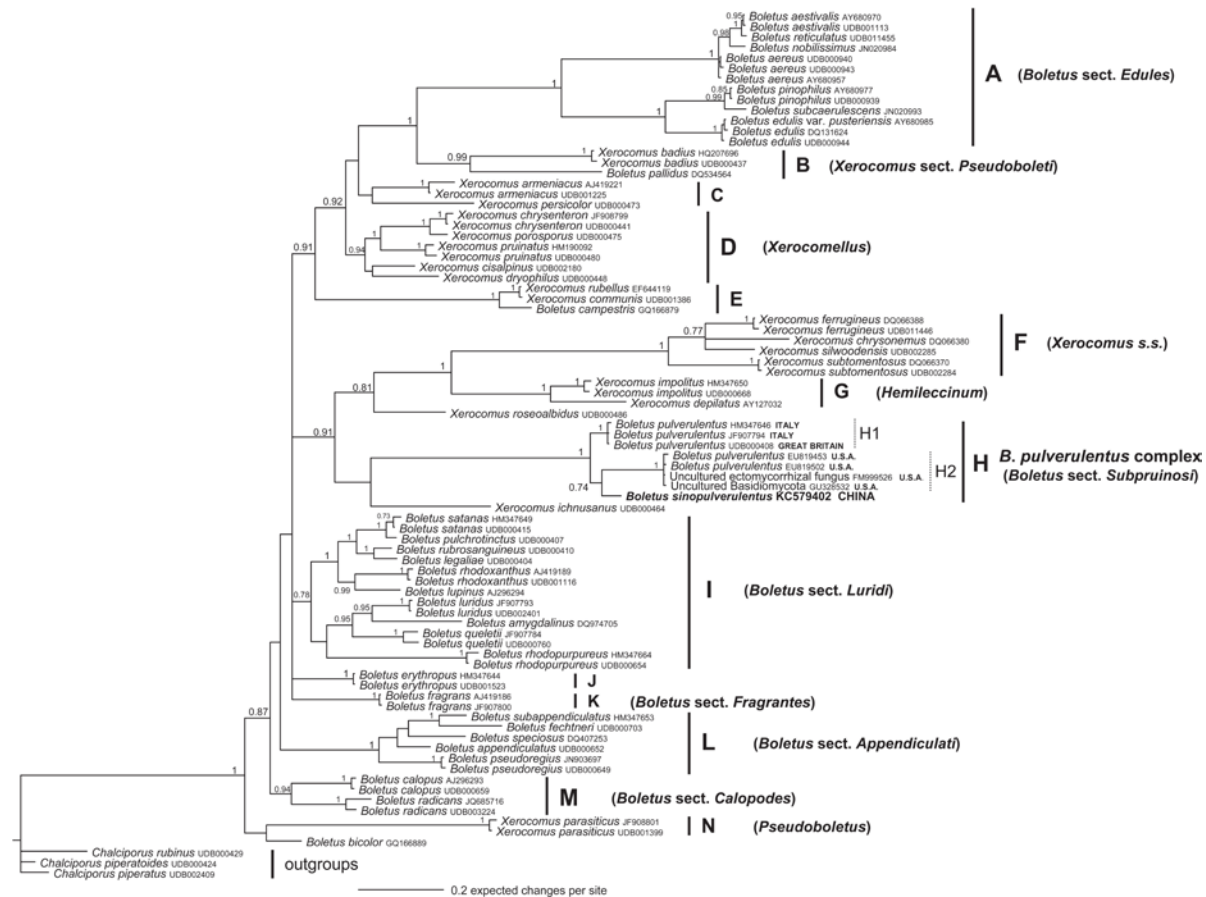


Fig. 1. Bayesian phylogram obtained from the ITS1-5.8S-ITS2) sequence alignment of *Boletus* and *Xerocomus* spp. BPP values over 0.70 are given above branches.

MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were performed using Geneious v5.3 (Drummond *et al.* 2010). The sequence is deposited in GenBank under the accession number given in Fig.1.

Sequence alignment and phylogenetic analysis

Sequences included in the phylogenetic analyses were either generated in this study or retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>) and UNITE (<http://unite.ut.ee/index.php?e0true>) databases. Alignments were generated using MAFFT (Kato *et al.* 2002) with default conditions for gap openings and gap extension penalties. The sequence alignments were slightly refined manually with MEGA 5.0 (Tamura *et al.* 2011). *Chalciporus piper-atooides* (UDB000424), *C. piperatus* (UDB002409) and *C. rubinus* (UDB000429) were used as outgroup taxa. Phylogenetic analysis was performed using the Bayesian Inference (BI) approach. The BI was performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) with four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC) run over 10 million generations, under GTR + Γ_4 evolutionary model. Trees were sampled every 1000 generations resulting in an overall sampling of 10001 trees; the first 2500 trees were discarded as "burn-in" (25 %). For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian Posterior Probabilities (BPP). Only BPP values over 0.70 are reported in the resulting tree (Fig. 1). Branch lengths were estimated as mean values over the sampled trees. Pairwise % identity values of ITS sequences (P%IV) were calculated using MEGA 5.0 (Tamura *et al.* 2011).

Results

Phylogenetic analysis

The ITS data matrix comprises a total of 83 sequences (one generated in this study, 42 from GenBank and 40 from UNITE). This dataset is 1150 base pairs long and contains 728 (63.3 %) variable sites. In the obtained Bayesian phylogram (Fig. 1), 14 well-supported major clades, A–N, were distinguished within the *Boletus-Xerocomus* assemblage, many of them agree with sub-specific taxa as traditionally delimited by Mu:foz (2005) and Sutara (1991, 2005, 2008). The sequence from our Chinese collection falls in the clade H (BPP = 1) consisting of subclade H1 (BPP = 1, three *B. pulverulentus* collections from Europe) and subclade H2 (BPP = 1, four *B. pulverulentus* collections from United States, EU819453 from basidiomata, EU819502 and FM999526 from ectomycorrhizal root tips, GU328532 from mycelia in soil). The P%IV of the entire clade H is 89.9; the European and the North American sequences show a P%IV of 98.7 and 99.5, respectively. The clade H is hereafter referred as the *B. pulverulentus* complex.

Taxonomy

Boletus sinopulverulentus Gelardi & Vizzini **sp. nov.** – Figs. 2–7.

MycoBank no.: MB 803339

Basidiomata small; pileus surface dry, matt, finely tomentose, evenly dark brown; hymenophore tubular, adnate to subdecurrent, deep yellow bruising dark blue; stipe surface dry, concolorous with the pileus, transversely streaked-scissurate in the upper half, not rooting; reticulum, partial veil and annulus absent; basal mycelium whitish; context whitish in the pileus to very pale yellowish in the stipe and reddish at the base, turning instantly indigo blue lengthwise on cutting, taste mild; spores medium-sized, $(9.0)12.5 \pm 1.00(14.3) \times (4.8)5.4 \pm 0.28(6.0) \mu\text{m}$, $Q = 2.31 \pm 0.14$, elliptical to ellipsoid-fusiform, smooth-walled, bright yellow in water and KOH, inamyloid or very faintly dextrinoid, basidia predominantly 2-spored, hymenophoral trama bilateral-divergent of the “*Boletus*-type”, stipe surface almost sterile but with lateral stipe stratum, inamyloid trama, clamp connections absent. Habitat: in temperate montane environment under *Castanea mollissima* Blume.

Type locality. – CHINA, Shaanxi Province, Qinling Mt., Heihe National Natural Forest Park, Yingbanliang village, 1432 m a.s.l., 30 September 2011; *leg.* M. Gelardi and J.-Z. Sun (holotype HMAS 266894; isotypes TO HG2821 and MG434a).

Etymology. – The specific epithet is a combination of Medieval Latin “sino” (which means “Chinese”) and “pulverulentus”, referring to the strong affinity of the new Chinese species to the European *B. pulverulentus*.

Basidiomata small. – Ontogenetic development gymnocarpic. – **Pileus:** up to 50 mm wide, flattened and faintly depressed at disc, regularly shaped, scarcely fleshy (context up to 6 mm thick in the centre), firm; margin curved downwards, even to hardly undulate, exceeding beyond the tubes up to 0.5 mm.; surface dry, matt, very slightly tomentose, not cracked, uniformly dark brown (Mahogany Red, Bay, Auburn, Plate II) and further but slowly darkening on handling; subcuticular layer whitish. – **Tubes:** thin and as long as the pileus context thickness (up to 4 mm), adnate and slightly decurrent on the stipe apex with a tooth, deep yellow (Empire Yellow, Plate IV) and immediately staining dark blue (Berlin Blue, Plate VIII) on cutting. – **Pores:** with a plain surface, somewhat small (up to 0.8 mm in diam.), simple, roundish, concolorous with the tubes and quickly turning dark blue (Berlin Blue, Plate VIII) then sordid brown on pressure; rusty brown spots are detectable at pores orifice. – **Stipe:** up to 60 x 12 mm, slightly off-centre, a little longer than pileus diameter, straight although curved at the base, solid, cylindrical but gradually enlarged from half-way down, rounded at the base and not rooting; surface dry, without reticulum, evenly dark brown (Ox-blood Red, Maroon, Plate I) with brownish-pink shades (Dahlia Purple, Plate XII) at the base, finely transversely streaked-scissurate in the upper half, the yellowish ground colour being visible in the cracks, bluing then slowly darkening when injured; partial veil and annulus absent; basal mycelium whitish. – **Context:** firm textured in the pileus, more fibrous in the stipe, whitish in the pileus, very pale yellow in the stipe but purplish-red (Dahlia Purple, Plate XII; Magenta, Plate XXVI) at the ex-

treme base, instantly and strongly bluing (Berlin Blue, Plate VIII) on exposure throughout and later fading to drab yellow; subhymenophoral layer whitish. – Smell: agreeable, fruity. – Taste: mild.

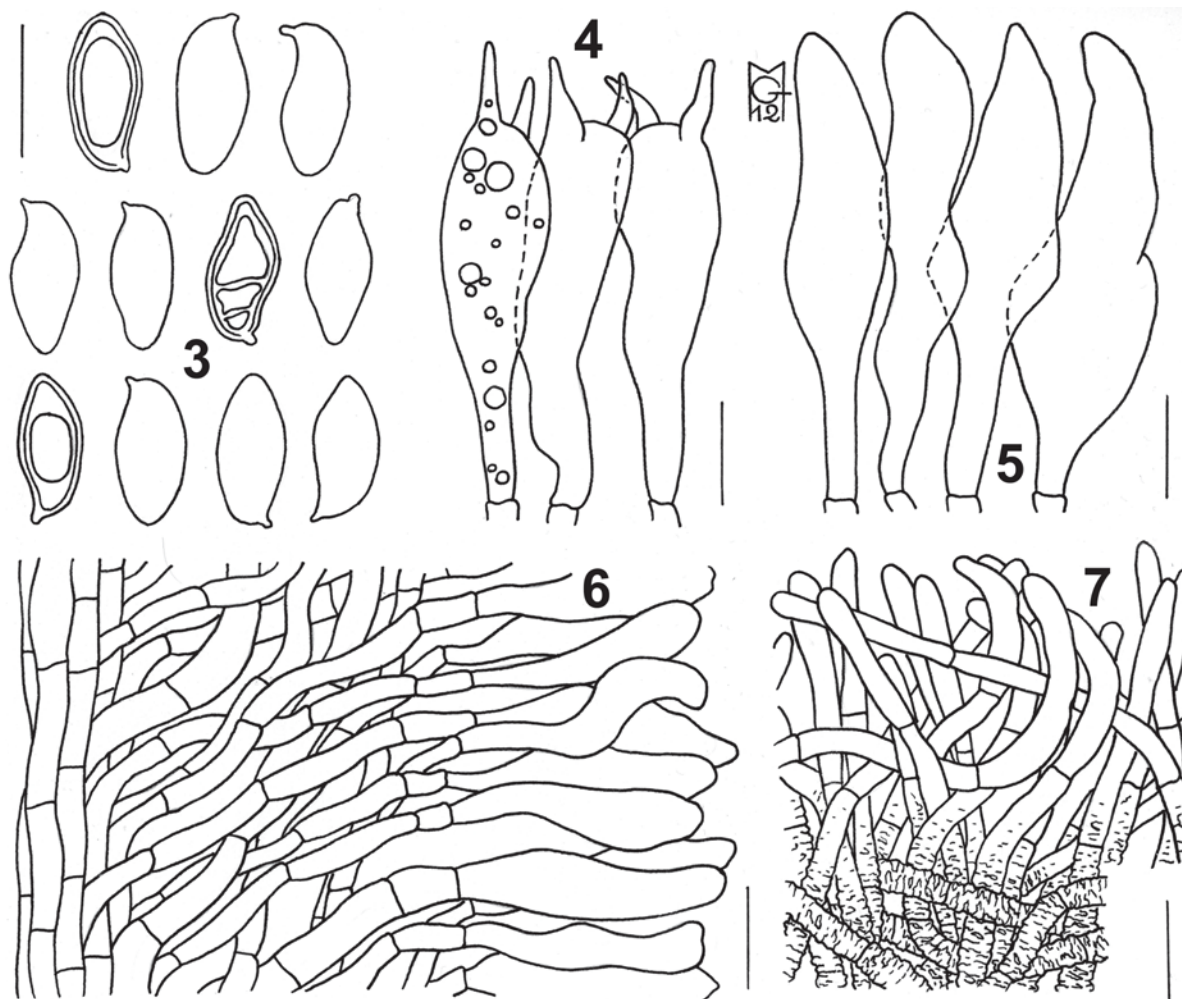
Spores: $[50,1,1](9.0)12.5 \pm 1.00(14.3) \times (4.8)5.4 \pm 0.28(6.0) \mu\text{m}$, $Q=2.31 \pm 0.14$, $V=194 \pm 32.0 \mu\text{m}^3$, asymmetric, elliptical to ellipsoid-fusiform in side view, elliptical in face view, smooth, with a long apiculus and a markedly pronounced supra-apicular depression, often very faintly constricted to-



Fig. 2. *Boletus sinopulverulentus*. Basidiomata in habitat (from HMAS 266894, holotype). Bar: 20 mm. Photo by M. Gelardi.

wards the apex and with rounded tip, moderately thick-walled ($0.5\text{--}0.8 \mu\text{m}$), bright yellow coloured in water and 5 % KOH, having one large or more rarely two to three oil guttules when mature, inamyloid or very faintly dextrinoid, acyanophilic and showing no metachromatic reaction. – Basidia: $(28)33\text{--}44(52) \times (9)10\text{--}13 \mu\text{m}$ ($n=11$), cylindrical to cylindrical-clavate, moderately thick-walled ($0.6\text{--}0.8 \mu\text{m}$), predominantly 2-spored but also 1- or 3-spored, very rarely 4-spored, usually showing long sterigmata (up to $8 \mu\text{m}$ long), subhyaline to bright yellow in water and 5 % KOH, containing straw-yellow oil guttules, without basal clamp connections; basidioles clavate. – Cheilocystidia: $(39)40\text{--}55(59) \times (6)9\text{--}11(13) \mu\text{m}$ ($n=10$), unfrequent, straight to less commonly flexuous, cylindrical-fusiform to more rarely

widely fusiform, occasionally lanceolate, with rounded apex, smooth, moderately thick-walled ($0.6\text{--}0.8\ \mu\text{m}$), hyaline to pale yellow in water and 5 % KOH, without epiparietal incrustations. - Pleurocystidia: (39)41-49(60) \times (8)12-14 μm ($n=8$), similar in shape, colour and size with cheilocystidia, unfrequent to rather rare. - Pileipellis: a trichodermium consisting of strongly interwoven, long, filamentous and sinuous, rarely branched hyphae without clamp connections at septa, tending to repent in the outermost layer



Figs. 3–7. *Boletus sinopulverulentus*. Microscopic features (from HMAS 266894, holotype). **3** Spores. **4** Basidia. **5** Pleuro- and cheilocystidia. **6** Lateral stipe stratum and stipitipellis. **7** Pileipellis. Bars: **3–5** 10 μm ; **6–7** 15 μm . Line drawings by M. Gelardi.

and thus turning into a cutis partially embedded in a gelatinous matter; terminal elements long and slender, cylindrical, with rounded or more rarely pointed apex, $(1.5)3.0\text{--}8.5(9.0)\ \mu\text{m}$ wide and up to 83 μm long, smooth walled, hyaline to straw yellow-brownish in water and 5 % KOH; subterminal elements similar in shape, size and colour with terminal ones; subpellis hyphae showing a coarse epiparietal brownish incrustation well detectable in Congo Red. - Stipitipellis: a texture of slender, parallel to loosely intermingled and longitudinally arranged, smooth-walled, adpressed hyphae, $(1.5)2\text{--}8(10)$

µm wide, hyaline in water and 5 % KOH; the stipe apex is covered by a layer of clavate to more rarely cystidioid sterile cells (caulocystidia), sometimes bifurcate at the tip, with rounded apex, 26-52 x 5-12 µm, pale yellow to golden yellow in water and 5 % KOH; fertile caulobasidia extremely rare. Lateral stipe stratum under caulohymenium present and well differentiated from the stipe trama, of the "boletoid type", at the stipe apex a 30-60(70) µm thick layer consisting of divergent, inclined and running towards the external surface, loosely intermingled and branched hyphae not touching each other and clearly embedded in a gelatinous substance. – Stipe trama: made up of longitudinally and densely arranged, subparallel to moderately interwoven, filamentous, smooth, inamyloid hyphae, 3-15 µm wide. – Hymenophoral trama: bilateral-divergent of the "*Boletus*-type", with somewhat divergent and loosely arranged, gelatinized hyphae (lateral strata hyphae in transversal section not touching and (3)4-10(12) µm distant from each other), hyaline in water and 5 % KOH; lateral strata 30-50 µm thick, mediostratum (20)25-40 µm thick, consisting of a tightly adpressed, not gelatinous bundle of hyphae, (1)2.5-9 µm wide; in Congo Red the mediostratum is darker than the lateral strata. – Clamp connections: none. – Hyphal system: monomitic.

Habitat. – In temperate montane environment, under a single tree of *Castanea mollissima* Blume standing in open countryside, on very moist and drained soil.

Distribution. – So far known only from the type locality (Shaanxi Province, China).

Material examined (besides type). – *Boletus pulverulentus*: ITALY, Lombardy, Cevo (BS), 1240 m a. s. l., 16 August 2008, *leg. & det.* M. Gelardi (MG126a); Lazio, Appia Antica Regional Park (Rome), 56 m a. s. l., 25 September 2009, *leg. & det.* M. Gelardi (MG237a); Lazio, Manziana (Rome), 320 m a. s. l., *leg.* M. Gelardi, L. Nicoletti and V. Migliozzi, *det.* M. Gelardi (MG460a); PORTUGAL, Azores Islands, São Miguel, *leg. and det.* T. Lezzi, 09 October 2011, TL20111009 (dupl. in herb. MG456a).

Discussion

Notes on the taxonomic status of *Boletus* and *Xerocomus*

Recent molecular research revealed that morphologically delimited large boletoid genera (Muñoz 2005, Kibby 2011, Knudsen & Taylor 2012), such as *Boletus* and *Xerocomus*, were most likely polyphyletic (Binder & Hibbett 2006, Dentinger *et al.* 2010, Li *et al.* 2011, Feng *et al.* 2012, Halling *et al.* 2012a,b, Zeng *et al.* 2012). Consequently, based on morphological and/or molecular data some taxa were recently segregated in new independent genera (e.g. *Hemileccinum* Šutara and *Xerocomellus* Šutara, Šutara 2008; *Austroropile* Halling & Fechner, *Harrya* Halling, Nuhn & Osmundson and *Sutorius* Halling, Nuhn & Fechner, Halling *et al.* 2012a, b; *Corneroboletus* N.K. Zeng & Zhu L. Yang, Zeng *et al.* 2012). Our data are in agreement with these authors; according to the ITS Bayesian analysis (Fig. 1), *Boletus* and *Xerocomus* are shown to be a heterogeneous consortium, an artificial assemblage.

where it is possible to detect 14 well-supported clades most of which match quite well with pre-existing morphologically-based supraspecific taxa (Lannoy & Estadès 2001, Muñoz 2005, Šutara 2008). In particular, clade A corresponds to *Boletus* sect. *Edules* Fr. (= *Boletus* sect. *Boletus*, = *Boletus* s. str., type *B. edulis* Bull.); clade B to *Xerocomus* sect. *Pseudoboleti* Singer (type *B. badius* Fr.); clade D to the genus *Xerocomellus* Šutara pro parte (type *B. chrysenteron* Bull.) (Šutara 2008, Kľofac 2011); clade F to the genus *Xerocomus* s. str. (type *B. subtomentosus* L.); clade G to the genus *Hemileccinum* Šutara (type *B. im-politus* Fr.) (Šutara 2008); clade H to *Boletus* sect. *Sub-pruinosi* Fr. emend. Singer (typus *B. barlae* Fr.); clade I to *Boletus* sect. *Luridi* pro parte (type *B. luridus* Schaeff.); clade K to *Boletus* sect. *Fragrantes* Lannoy & Estadès (type *B. fragrans* Vittad.); clade L to *Boletus* sect. *Appendiculati* Konrad & Maubl. emend. Lannoy & Estadès (typus *B. appendiculatus* Schaeff.); clade M to *Boletus* sect. *Calopodes* Fr. emend. Lannoy & Estadès (type *B. calopus* Pers.); clade N to the genus *Pseudoboletus* Šutara (= *Xerocomus* sect. *Parasitici* Singer, type *B. parasiticus* Bull.) (Šutara 1991). *Xerocomus armeniacus* (Quél.) Quél./*X. persicolor* H. Engel, Kľofac, H. Grünert & R. Grünert (clade C), *Xerocomus rubellus* (Krombh.) Quél./*X. communis* (Bull.) Bon/*Boletus campestris* A. H. Sm. & Thiers (clade E), *Xerocomus roseoalbidus* Alessio & Littini, *Xerocomus ichnusanus* Alessio, Galli & Littini, *Boletus erythropous* Pers. (clade J), and *Boletus bicolor* Peck seem to occupy an isolate position.

Further studies including additional gene sequences are likely to lead to major changes in the generic concept of this assemblage in the future. Our data suggests that most of these well supported monophyletic lineages within *Boletus* and *Xerocomus* deserve to be raised to the status of distinct genera.

The *Boletus pulverulentus* complex

Boletus sinopulverulentus clusters, as independent species, as sister to *Boletus pulverulentus* sequences from United States (subclade H2) (Fig. 1); they are in turn sister to *B. pulverulentus* sequences from Europe (subclade H1). The most reliable characters for distinguishing *B. sinopulverulentus* can be summarized as follow: (1) small dimension and frail habit; (2) dark brownish tints on both pileus and stipe; (3) yellow pores; (4) stipe without reticulum but finely scabrous-scissurate radially, resembling a zebra-pattern; (5) basidiomata quickly turning indigo blue on handling and exposure; (6) whitish basal mycelium; (7) mild taste; (8) ellipsoid-fusiform, smooth walled, bright yellow spores; (9) predominantly 2-spored basidia; (10) bilateral-divergent “*Boletus*-type” hymenophoral trama; (11) almost completely sterile stipe surface; (12) presence of lateral stipe stratum; (13) inamyloid trama.

The remarkable dark blue-black staining European *B. pulverulentus* is undoubtedly the most closely related species from the morphological viewpoint, differing from the Chinese taxon on the basis of the pruinose and peculiarly coloured stipe which is of a bright yellow in the upper half but more

or less sharply reddish to brownish-red from the middle zone downwards and instantly bruising deep ultramarine blue on handling, the rooting stipe, the slightly longer and narrower spores with a Q value of 2.6–2.9 and the shorter and wider 4-spored basidia (Pilat & Dermek 1974, Engel *et al.* 1983, Alessio 1985, Breitenbach & Kränzlin 1991, Urbonas 1997, Lannoy & Estadès 2001, Muñoz 2005, Watling & Hills 2005, Galli 2007, Kłofac 2007,

Knudsen & Taylor 2008, 2012, Šutara *et al.* 2009, Eyssartier & Roux 2011, Kibby 2011; pers. obs.). Interestingly, the nearly sterile stipe surface of *B. sinopulverulentus* is apparently similar to that of *B. pulverulentus* in which the sporulating caulobasidia are said to be constantly present but very difficult to observe due to the occurrence of an amorphous incrustation (Bessette *et al.* 2000, Šutara 2005). In the former species, however, such incrustation has not been observed and caulobasidia are indeed extremely rare. Nonetheless the lateral stipe stratum under the stipitipellis is well developed and easily detectable unlike most part of the *Boletaceae* Chevall. with a sterile stipe which never show this feature (Šutara 2005). In addition, both species lack reticulation on the stipe, even though *B. pulverulentus* may occasionally exhibit a very short and barely defined reticulum immediately below the junction point with the tubes (Lavorato & Simonini 1997, Muñoz 2005) and an American phenotype from Tennessee, *B. pulverulentus* f. *reticulatus* Snell, Dick & Hesler has been described as displaying a well developed reticulated stipe (Bessette *et al.* 2000). *Boletus pulverulentus* var. *sublateritius* Guinberteau, Lannoy & Estadès *ad interim* from France also differs by the carmine red pileus (Lannoy & Estadès 2001).

Although *B. pulverulentus* from USA might appear conspecific with the European taxon based on morphology alone (Smith & Thiers 1971, Bessette *et al.* 2000), the present study demonstrates it to be different based on molecular inference (Fig. 1). Future work will be focused on describing this new taxon.

When compared to *B. sinopulverulentus*, the North American *B. rainisii* A. E. Bessette & O. K. Miller differs in having a dark olivaceous-brown pileus becoming rimose in age, bright yellow glabrous stipe with reddish hues at the extreme base, yellow context, longer spores and growth in association with conifers (Bessette *et al.* 2000).

Up to now only one locality in central China is known for the occurrence of *B. sinopulverulentus* but it might have been confused in the past with the widespread and more common *B. pulverulentus* s. l. which has also been reported from China (Zang 1996, Li & Song 2000, Mao 2009).

In accordance with the taxonomic arrangement outlined by Singer (1986) and Muñoz (2005), *B. sinopulverulentus* is at present tentatively placed in sect. *Subpruinosi* (basidiomata with a *Xerocomus*-like habit and stipe without reticulum), however, a comprehensive molecular revision of *Boletus* s. l. worldwide is urgently required in order to provide further insights and definitely assess the taxonomic status of *B. sinopulverulentus* and its allied species within *Boletaceae*.

Acknowledgements

Very special thanks go to the State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, for inviting and supporting MG and providing the fieldwork opportunities. The first author is also indebted to T. Lezzi (Rome, Italy) who provided a sample of *B. pulverulentus* from Azores Islands.

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